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(54) Method of assay for hemoglobin in feces

(57) Haemoglobin in faeces is assayed by a method comprising physically adsorbing haemoglobin present in faeces onto the surface of a sampling stick formed of a hydrophobic high molecular weight material to separate the haemoglobin from the faeces and then specifically detecting the haemoglobin with enzyme-labelled anti-human haemoglobin.

The sampling stick may have a rough or grooved surface sample-taking portion and a flat handle portion for identification data.

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FIG. 1

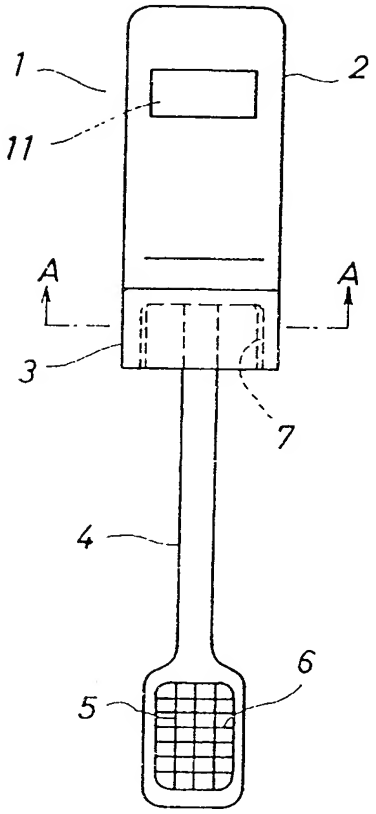


FIG. 2

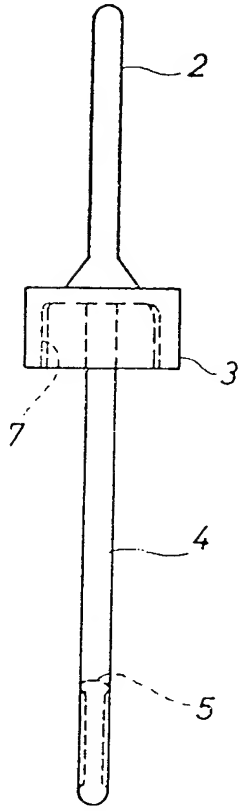


FIG. 3

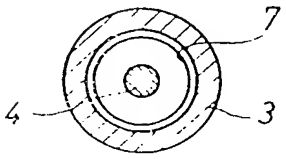


FIG. 4

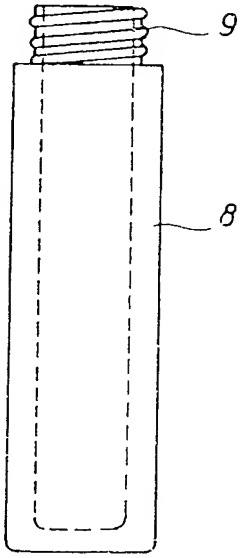
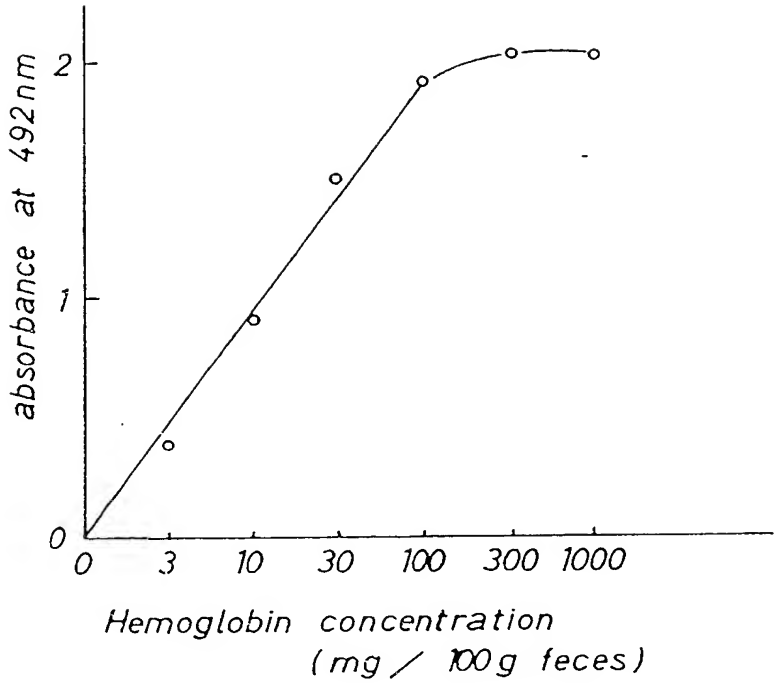


FIG. 5


absorbance at 492nm
2
1
0
0
3
10
30
100
300
1000
Hemoglobin concentration
(mg / 100g feces)

SPECIFICATION

Method of assay for hemoglobin in feces

The present invention relates to an assay method for hemoglobin in feces and more particularly, to a method of assay capable of presuming pathologic regions in the digestive organ system without any requirement on the subjects (or patients) for dieting, etc. and capable of assaying a large number of samples as in mass examination, etc. in a simple manner.

An assay method for hemoglobin (blood) in feces has been performed for purposes of early detection of abnormalities, e.g., cancer, ulcer, etc., in the digestive organ system.

For the assay for hemoglobin in feces, chemical detection of fecal occult blood has been employed heretofore. In recent years, frequency attacked with colorectal cancer is markedly increasing in advanced countries. Namely, eating habits have been westernized, resulting in an increased rate of the outbreak of colorectal cancer. For this reason, chemical detection of fecal occult blood have been widely conducted as mass examination for detection of colorectal cancer at early stages in view of simplicity and low costs. The chemical detection of fecal occult blood is very sensitive so that even a small quantity of hemoglobin can be detected.

However, the prior art chemical detection of fecal occult blood has no specificity to human hemoglobin since the tests are based on a colorimetric reaction with various chromogens based on peroxidase activity contained in hemoglobin of erythrocyte. Therefore, the reaction occurs also with peroxidases in animal hemoglobin-, myoglibin- or chlorophyl- containing vegetables (broccoli, radish, califlower, carrot, cucumber, pumpkin, cabbage, etc.) of non-human animals. In addition, the tests are affected by administered medicines (iron, copper, potassium iodide, vitamin C, etc.). For increasing the specificity to human hemoglobin, the tests thus encounter a problem that diet control such as abstaining from meat dishes, etc., is required prior to examination. In actual mass examination, a false-positive increase is prevented by diet control and at the same time, using chemical detection of occult blood in which a chromogen having a low sensitivity is used. As a result, however, there is a danger of conversely increasing false-negative (even when hemoglobin is present, the hemoglobin is not detected).

Further in the chemical detection of fecal occult blood in the prior art, bleeding is detected in the whole digestive organ path from the oral cavity to the anus because both hemoglobin in fresh blood and hemoglobin decomposed with digestive enzyme exhibit peroxidase activity; accordingly, exact information about the bleeding region is not obtained. It is thus impossible to satisfy both specificity and sensitivity for proving bleeding from the lower region of the digestive organ system, for example, colorectal cancer, etc.

Due to the foregoing shortcomings, mass examination by chemical detection of fecal occult blood has not been established yet.

On the other hand, a method for immunological detection of fecal occult blood using antibody-antigen reaction between human hemoglobin and anti-human hemoglobin has recently been developed by Barrow et al (Am. J. Cli. Pathol., 69: 342, 1978, March). The immunological detection of fecal occult blood is characterized in that hemoglobin loses most of its antigenicity by the action of digestive enzyme in the digestive organ system so that not only the specificity to human hemoglobin but also lower gastrointestinal bleeding only which retains antigenicity can be detected.

However, assay methods developed to date based on the principle of the immunological occult blood require operations for taking a fixed amount of feces, mixing it with an aqueous medium, then centrifuging the mixture to obtain the supernatant, which is used as a sample. In addition, in the single radial immuno-diffusion method (in which a sample is diffused in a gel containing antibody to examine the reaction) and the Ouchterlony method (in which antibody and a sample are injected in a gel at fixed intervals, both are diffused to react with each other, and the reaction is examined), a relatively long period of time such as 24 to 48 hours is required for judgment of the results. Further the counter immunoelectrophoresis method (wherein the reaction product between a sample and antibody is measured on a carrier by applying an electric current) involves disadvantages that special equipments for analysis are required, etc. and therefore, has not come to be widely used. Furthermore, these methods are not suitable for mass examination handling a large number of samples.

In order to apply this test to mass examination, it is necessary that a large number of fecal specimens be taken in a fixed amount, respectively, conveyed to and handled in an analysis room with ID (identification) thereon. This procedure also involves a disadvantage unpleasant to the operator due to an offensive smell, uncleanness, etc.

In view of such actual situations, we have extensively investigated on a method of screening by mass examination of gastrointestinal bleeding such as colorectal cancer, with an attempt to overcome the foregoing disadvantages associated with the prior art chemical detection of fecal occult blood and immunological fecal occult blood test. As a result, we have found that hemoglobin in feces is easily physically adsorbed to the surface of a high molecular material, particularly hydrophobic plastic resin. By utilizing this phenomenon

we have been successful to easily separate hemoglobin from feces on the surface of a plastic resin article (e.g. sampling stick). We have also found that the physically adsorbed hemoglobin in such a way can be used for an enzyme immuno-assay for the detection of fecal human hemoglobin wherein is used enzyme-labeled anti-human hemoglobin, whereby only human hemoglobin can be specifically detected with a high sensitivity.

Thus the present invention provides a method of assay for hemoglobin in feces which comprises physically adsorbing hemoglobin in feces to the surface of a sampling stick made of a hydrophobic high molecular material to separate the hemoglobin from feces and specifically detecting the hemoglobin with the use of enzyme labeled anti-human hemoglobin.

One of the important features of the present invention is therefore to use a sampling stick made of a hydrophobic high molecular material. Examples of such high molecular materials are polystyrene, polyvinyl chloride, polyethylene, polypropylene, polycarbonate, acrylic resins, etc., among which polystyrene is preferable.

In carrying out the method of the present invention, the sampling stick is contacted with feces to be tested so that hemoglobin in the feces is physically adsorbed on the surface of the sampling stick. Then, preferably, the stick is washed with water to remove any feces. Then the sample (hemoglobin) adsorbed on the stick is subjected to antibody-antigen reaction by the use of an enzyme-labelled anti-human hemoglobin and subsequent colorimetric reaction for the determination of hemoglobin.

These reactions may be conducted on the sampling stick itself or in a separate reaction vessel. Thus the sampling stick with physically adsorbed hemoglobin thereon is immersed in or dropwise added with an enzyme-labelled antibody solution to conduct the antigen-antibody reaction on the sampling stick. Thereafter the sampling stick with the hemoglobin thereon as reacted with the enzyme-labelled antibody is immersed into or added with a solution containing a substrate for color development so as to cause a colorimetric reaction. Then the human hemoglobin in feces is specifically detected colorimetrically.

The enzymatic immuno reaction is well known in the art. For general information thereof, reference may be made, for example, to U.S. Patent No. 4,016,043. The colorimetric reaction for assay of this kind is also well known in the art, e.g. Kind-King method (J. Clin. Path. 7 322–326, 1954).

If desired, in addition to the enzymatic immuno reaction method, it is also possible to conduct a conventional chemical detection method in respect of the hemoglobin as physically adsorbed on the sampling stick. When such chemical detection method (chemical detection of fecal occult blood) is conducted, in addition to the enzymatic immuno assay, there can be assured the detection of hemoglobin in feces more accurately, and at the same time, it becomes possible to presume the region of bleeding in the digestive system, because the hemoglobin physically adsorbed to the surface of the hydrophobic high molecular material is also applicable to such prior art chemical detection of fecal occult blood. When a chemical detection of fecal occult blood is conducted with the hemoglobin separated as adsorbed on a hydrophobic high molecular material, interference of vegetable peroxidases and medicinal chemicals is eliminated, but hemoglobin or myoglobin derived from animal (ingested meat) is detected as in the prior art.

In case such chemical detection method is carried out, it is preferable to employ an acidic buffer in the operation for the hemoglobin adsorption on the surface of a sampling stick, because hemoglobin will be changed in the chemical structure by the action of such acidic buffer so that it becomes more easily adsorbed on the surface of a hydrophobic high molecular material. Thus, for example, the sampling stick with feces thereon is immersed in or added with an acidic buffer so that hemoglobin will be physically adsorbed on the surface of the sampling stick with a more certainty over a wide range of concentration. Therefore, when such chemical detection method is employed the detection of hemoglobin even in a low concentration in feces becomes possible. However, when subjected to the action of an acidic buffer, the hemoglobin will get out of its configuration (stereo structure) so that the acid treatment is sometimes not preferable for the enzymatic immuno reaction method.

The chemical detection method for hemoglobin (or chemical detection of fecal occult blood) is well known in the art. For details thereof reference may be made, for example, to "Rinsho Kensa" vol. 7, No. 2, pages 70–71 (1963).

The invention will be further explained as follows by referring partly to the attached drawings wherein:

Figure 1 is a front view of a sampling stick which may be used in this invention,

Figure 2 is a side view of the sampling stick,

Figure 3 is a cross-sectional view taken along the line A–A of Fig. 1,

Figure 4 is a front view of a vessel which may be used in association with the sampling stick, and

Figure 5 is a graph showing the relation between the hemoglobin concentrations in various feces tested and the absorbances at 492 nm obtained by the detection according to this invention.

In carrying out the method of this invention

there may be used a sampling stick (made of a hydrohobic high molecular material) of any proper shape or consutruction so far as it has a sample-taking surface which can contact with feces to be tested to physically adsorb thereon hemoglobin in the feces. An example of a sampling device is shown in Figs. 1–4. As shown, the device comprises a sampling stick 1 and a liquid vessel 8. The sampling stick 1 has a body 3 with an inside-threaded cavity 7, equipped with a flat handle 2 on which ID (identification) label 11 for the subject (or patient) may be applied. At the bottom of the cavity 7 there is provided a rod 4 extending outwardly and having a sample-taking portion 5 at the end. It is preferable that the sample-taking portion 5 has a rough or coarse surface or is formed with fine grooves 6, preferably lattice-like, to increase the area of contact with feces or to ensure retention thereon of the sample or any treating liquid added thereto. It is further preferable that the sample-taking portion 5 is so shaped or flattened that both surfaces may be utilized for sampling. When the colorimetric reaction is to be conducted on the sampling stick itself it is preferable that the sampling stick is made of a white material in order to facilitate the examination of the result of the colorimetric reaction. The structure of the sampling stick is not limited to that shown, and may be of a spoon-like structure at the edge thereof or may be of structure in which feces is collected only at one surface thereof.

If desired the sampling stick 1 may be associated with a cylindrical vessel 8 provided with threads 9 engageable with the threaded cavity 7 of the sampling stick 1. In the vessel 8 there is contained an aqueous medium (e.g. deionized water, distilled water buffer solution), so that the hemoglobin adsorption antigen-antibody reaction and/or colorination reaction may be conducted therein.

For conducting the method according to this invention by the use of such device as shown in Figs. 1–4, the feces of the subject (or patient) to be tested is collected on the surface(s) of a sampling stick e.g. as shown in Figs. 1–3. Then the sampling stick 1 is inserted into the vessel 8 so that the sample-taking portion 5 with the feces thereon is immersed in the aqueous medium in the vessel 8. The sampling stick 1 and vessel 8 may be fixed together by means of threads 7 and 9. Before inserting the sampling stick 1 into the vessel an ID label 11 may be applied to the handle 2.

The physical adsorption of hemoglobin in the sampled feces can be effected by allowing the sampling stick with the feces thereon to stand for a proper period of time e.g. several seconds to several minutes, preferably about 1 minutes. Preferably the adsorption operation is conducted in the presence of an aqueous medium e.g. in the vessel 8 as explained above. Thereafter the sampling stick is taken out and the sample-taking portion is washed with distilled or deionized water.

According to the method, for example, feces are taken and a vessel assembled with a sampling stick is allowed to stand in a lavatory. Then the vessel is disassembled after a fixed period of time and only the sampling stick can be conveyed as a sample to a place where subsequent assay is conducted. Thus only hemoglobin is adsorbed to the sampling stick which is free from unpleasant odors or dirtiness so that the operator is free from an unpleasant feeling and, collection and conveyance are easy.

Then the sampling-stick with hemoglobin adsorbed thereon is subjected to enzyme immunoassay and determination (measurement) of human hemoglobin. These methods are known per se as mentioned hereinbefore and therefore only a brief explanation will be made as follows for typical exemplification.

A. Preparation of reagent for enzyme immunoassay:

(1) Preparation of hemoglobin A_o:

Hemoglobin A_o is prepared from human blood in accordance with, e.g., the method of Williams et al (Anal. Biochem., 54: 137, 1973).

(2) Preparation of anti-human hemoglobin:

Purified hemoglobin A_o is mixed with Freund's complete adjuvant and animals such as rabbits, goats, etc. are immunized with the mixture. Blood is collected from the immunized animal and centrifuged to obtain antiserum. Rabbit anti-human hemoglobin is commercially available (e.g. product of DAKO-PATTS, Denmark) and it is used for enzyme-labelling.

(3) Labelling with enzyme:

Anti-serum is labelled with enzyme according to, e.g., the method of Yoshitake et al (Immunological Experimental Method XI, pages 3497–3519, 1982, published by The Society of Immunology, JAPAN) to prepare enzyme-labelled antibody. In this case, various enzymes such as peroxidase, alkaline phosphatase, etc. can be employed.

B. Method:

(1) The surface of a sampling stick to which hemoglobin bas been physically adsorbed is thoroughly washed with deionized or distilled water.

(2) After the enzyme-labelled antibody reagent is dropwise added to the surface or the sampling stick is immersed in the enzyme-labelled antibody reagent, the system is allowed to stand, e.g., at 20 to 40°C for 15 to 30 minutes to perform antigen-antibody reaction.

(3) Then the sampling stick is thoroughly washed with distilled or deionized water, a substrate solution (colorimetric reaction solution), containing e.g., p-nitrophenylphosphoric acid or 4-aminoantipyrine is dropwise added

thereto, or the sampling stick is immersed into such substrate solution to conduct the colorimetric reaction.

(4) After the completion of the colorimetric reaction the degree of color formation is observed and judged.

According to the method, a large amount of samples can be detected within about 1 hour.

The assay for hemoglobin in feces based on the enzyme immunoassay has been described hereinabove. When chemical detection of occult blood conventionally performed is used in addition to the above method, hemoglobin in feces can be detected more accurately. In such case it is preferable to employ a sampling stick 1 as shown in Figs. 1–3 and to use one surface of the sample-taking portion 5 for enzyme immuno assay method and the other surface for chemical detection method.

The invention will be further explained by means of the following Examples which are given for exemplification only and not for limitation of the scope of the invention.

EXAMPLE 1

Purified hemoglobin A_0 was mixed with Freund's complete adjuvant, with which rabbit was immunized once a week. An amount of the antigen used for each immunization was 0.5 mg, which was used after mixing with 2.5 ml of Freund's complete adjuvant. The imminization was repeated 5 times. On 5th day after the final immunization, blood was collected and centrifuged to obtain anti-sera (anti-human hemoglobin), and stored at −80°C in a frozen state.

Next, by the use of the above prepared anti-human hemoglobin, alkaline phosphatase-labelled anti-human hemoglobin (containing 0.2% of bovine albumin and 0.5 mM of magnesium chloride) was prepared as an enzyme-labelled antibody reagent, with the use of alkaline phosphatase.

Hemoglobin was adsorbed to a sampling stick as shown in Figs. 1–3 by contacting the stick with hemoglobin-containing feces for 1 minute. The sampling stick was thoroughly washed with deionized water and transferred to a small testing tube in which 0.3 ml of the enzyme-labelled antibody reagent was charged. Antigen-antibody reaction was performed at 37°C for 30 minutes. Then, the sampling stick was washed with deionized water and put into a small testing tube charged with 0.5 ml of a diethanolamine substrate solution (pH 10.5) containing 40 mM of p-nitrophenylphosphoric acid and 0.5 mM of magnesium chloride. Incubation was performed at 37°C for 5 to 10 minutes. Further 0.5 ml of 1N NaOH was added thereto to discontinue the reaction.

The degree of color formation (yellow in this case) was observed and judged with the naked eye.

As a result of the judgment of various samples, it was noted that samples showing the hemoglobin concentration of 0.5 mg/dl or more were obviously discernible to be positive and, in a high concentration, color formation of strongly positive occurs in the concentration of 300 mg/dl or more over the region reaching the hemoglobin concentration in whole blood.

REFERENCE EXAMPLE

In 1 ml of Bicine buffer (pH 8.5, product of Nakarai Chemicals, Ltd., Japan) containing 1 mM $MgCl_2.6H_2O$ there were dissolved 10 mg of alkaline phosphatase (product of Boehlinger). The solution was added with 20 mg of potassium periodate and stirred for 2 hours at 37°C. This solution was passed to a column (1.5×50 cm) of Sephadex G–25 (product of Pharmacia) bufferized (equilibrated) with 0.1 m carbonate buffer (pH 9.5) containing 1 mM $MgCl_2.6H_2O$ amd the protein fraction was collected. The protein fraction was added with 10 mg of rabbit anti-human hemoglobin (commercial product of DAKOPATTS, Denmark), and the mixture was reacted at 4°C for 24 hours under stirring. The resulting solution was passed to a column (1.5×75 cm) of Sephadex G–200 (product of Pharmacia) bufferized with 0.1 M carbonate buffer (pH 9.5) containing 1 mM $MgCl_2.6H_2O$, and the enzyme-labelled antibody fraction was collected.

EXAMPLE 2

1. Sampling:

A sampling stick as shown in Fig. 1 was inserted in and drawn out of feces to be tested. The operation was repeated at about ten different portions of the same sample feces. Different sample feces added with hemoglobin in different concentrations were used. Then, each sampling stick on which hemoglobin was adsorbed was washed with distilled water to wash the deposited feces away and lightly wiped with a soft tissue paper.

2. Reaction with enzyme-labelled antibody:

In 1.0 ml of a physiological buffer salt solution (pH 7.3, containing 1 mM $MgCl_2.6H_2O$) containing 0.2% bovine serum albumin, there were added 2μg of the enzyme-labelled antibody as prepared in REFERENCE EXAMPLE. The sampling stick was immersed into the solution to cause reaction for 15 minutes at 37°C. Then the sampling stick was taken out and well washed with distilled water.

3. Colorimetric reaction:

Then the above sampling stick was immersed into 1.0 ml of a substrate solution (prepared by dissolving 0.215 g of phenyl phosphate 2Na and 0.09 g of 4-aminoantipyrine in 0.05 M carbonate buffer of pH 10.5 to make 200 ml solution) to cause reaction for 15 minutes at 37°C. After taking the sampling stick away, the solution was added with 1.0 ml of a color developing solution (prepared by

dissolving 0.38 g of potassium periodate and 2.6 g of boric acid in 200 ml of water) for the colorimetric reaction at room temperature for 10 minutes. After the reaction the absorbance at 492 nm was measured. The colorimetric reaction was conducted on the basis of the Kind-King method (J. Clin. Path. 7 322–326, 1954).

4. Result:

The results are shown in Fig. 5 which shows the relation between the absorbances and hemoglobin concentrations in the various tested feces.

EXAMPLE 3

In the same manner as in Example 1, feces were collected on both surfaces of a sampling stick and, hemoglobin was physically adsorbed to the both surfaces of the sampling stick for a period of 30 seconds in the presence of a 1M citrate solution as a buffer solution.

After the sampling stick was thoroughly washed with deionized water, a reagent for chemical detection of occult blood containing o-tolidine hydrochloride and hydrogen peroxide in a 1M acetate buffer solution was dropwise added to one surface. After allowing to stand for about 10 minutes, a colorimetric (blue in this case) reaction was caused and judged with the naked eye. As a result of the experiment, it was noted that a positive result in the colorimetric reaction was obtained with hemoglobin of 1 mg/dl or more.

Immediately after completion of the chemical detection of occult blood, the back surface of the sampling stick was turned up and a drop of the enzyme-labelled antibody reagent used in Example 1 was applied thereto. The sample was allowed to stand at 20 to 40°C for 30 minutes.

After the sampling stick was thoroughly washed with deionized water, a substrate solution used in Example 1 was dropwise added to the said back surface of the sampling stick, i.e., the surface for enzymatic immune reaction. The colorimetric (yellow) reaction was evaluated 5 minutes after.

Also in this method, a positive result is obviously discernible with a hemoglobin concentration of 0.5 mg/dl or more; it is noted that in high concentrations, the color formation is strongly positive over the entire regions of 300 mg/dl or more, or reaching the hemoglobin concentration in whole blood.

In various experiments by this method, hemoglobin which underwent digestive reaction with pepsin and trypsin was incorporated in feces from normal healthy persons (free from hemoglobin) to examine the reactivity. The reactivity was positive in chemical detection of occult blood and negative in enzymatic immune reaction. Next, fresh blood was incorporated in the same feces to establish the staining conditions in the colon at 37 to 40°C and hemoglobin was detected there. The results were positive both in chemical detection of occult blood and in enzymatic immune reaction.

Thus it has been clarified that by the detection of hemoglobin in feces using enzymatic immune reaction along with chemical detection of occult blood, a source of hemoglobin could be presumed, either the upper part or the lower part of the digestive organ system.

As described in detail hereinabove, according to the method of assay for hemoglobin in feces by adsorbing hemoglobin in feces to the surface of the high molecular material of the present invention based on enzymatic immune reaction, drawbacks of widely used chemical detection of fecal occult blood from old, i.e., drawbacks in both specificity and sensitivity, can be solved and, operability can be extremely simplified as compared to other assay methods using the principle of conventional immunological reaction so that practical use of the assay based on immunological reaction can be greatly improved. Further from a clinical viewpoint, the present invention is useful as screening test for detection of colorectal cancer, etc. particularly with increasing outbreak recently in their early stages, because only lower gastrointestinal bleeding can be detected. The present invention is also advantageous in that operation for collecting samples is clean by utilizing adsorption of hemoglobin to the high molecular material.

In addition, by conducting the enzymatic immune reaction along with the chemical detection of fecal occult blood, occult blood in feces can be more accurately detected and at the same time, the effect of capable of presuming the bleeding part in gastrointestinal organs is exhibited from a clinical viewpoint.

CLAIMS

1. A method of assay for hemoglobin in feces which comprises physically adsorbing hemoglobin in feces to the surface of a sampling stick made of a hydrophobic high molecular material to separate the hemoglobin from feces and specifically detecting the hemoglobin with enzyme labelled anti-human hemoglobin.

2. A method of assay for hemoglobin in feces as claimed in claim 1 wherein chemical detection of occult blood is conducted in addition to the enzymatic immune reaction to detect the hemoglobin.

3. A method as claimed in any of claims 1 and 2 wherein the hydrophobic high molecular material is selected from the group consisting of polystyrene, polyvinyl chloride, polyetylene, polypropylene, polycarbonate and acrylic resins.

4. A method as claimed in any of the preceding claims wherein the sampling stick has a sample-taking portion which has a rough or course surface or which is formed with fine grooves.

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